Activation of mitogen-activated protein kinases (p38-MAPKs, SAPKs/JNKs and ERKs) by adenosine in the perfused rat heart

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Abstract Adenosine and mitogen-activated protein kinases (MAPKs) have been separately implicated in cardiac ischaemic preconditioning. We investigated the activation of MAPK subfamilies by adenosine in perfused rat hearts. p38-MAPK was rapidly phosphorylated and activated (10-fold activation, maximal at 5 min) by 10 mM adenosine, as was the p38-MAPK substrate, MAPKAPK2 (4.5-fold). SAPKs/JNKs were activated (5-fold) and ERKs were phosphorylated (both maximal at 5 min). The concentration dependences of activation of p38-MAPK and ERKs were biphasic with a 'high affinity' component (maximal at 10–100 μM adenosine) and a 'low affinity' component that had not saturated at 10 mM. SAPKs/JNKs were activated only by 10 mM adenosine. These results are consistent with MAPK involvement in adenosine-mediated ischaemic preconditioning.

Key words: Heart; Adenosine; Mitogen-activated protein kinase

1. Introduction

Adenosine (Ado) is formed in the heart by the hydrolysis of adenine nucleotides, a process that occurs extensively in ischaemia and reperfusion [1]. Ado may be an endogenous mediator of ischaemic preconditioning, where brief periods of sublethal ischaemia increase the heart's resistance to subsequent periods of more severe ischaemia [1–4]. Ado exerts its preconditioning effects, at least in part, through the activation of G_i protein-coupled Ado receptor A_1 and A_3 subtypes [1,3,4]. The signalling mechanisms responsible for ischaemic preconditioning are unclear and are the subject of intense investigation. Several groups have demonstrated that mitogen-activated protein kinase (MAPK) subfamilies are activated by ischaemic preconditioning protocols [5–7]. p38-MAPKs are activated during ischaemia and this is maintained during subsequent reperfusion. Stress-activated protein kinases (SAPKs), which are also known as c-Jun N-terminal kinases (JNKs), are activated during reperfusion following ischaemia. It is not clear whether a third group of MAPKs, the extracellularly responsive kinases (ERKs), is activated by ischaemic preconditioning. Recently, p38-MAPK has been directly implicated in ischaemic preconditioning [8] and here we have examined the regulation of all three MAPK subfamilies in the perfused heart by Ado.

2. Materials and methods

2.1. Heart perfusions

Adult male (275–325 g) Sprague-Dawley rat hearts were perfused retrogradely as described previously [9]. After a 15 min equilibration period, hearts were perfused with Ado for a further 2–30 min or with 1 μM phorbol 12-myristate 13-acetate (PMA) for 5 min. Hearts were also subjected to ischaemia/reperfusion as previously described [9]. Control hearts were perfused for up to 30 min following equilibration. Hearts were 'freeze-clamped' whilst still perfusing and pulverised under liquid N_2. Heart extracts were prepared using the extraction buffers described previously [9] and clarified by centrifugation ($10\,000 \times g$, 10 min). Protein concentrations were determined using the biuret method [10].

2.2. Protein kinase assays

For in gel kinase assays, proteins (60 μg) were separated on 10% (w/v) SDS-polyacrylamide gels with a 6% (w/v) stacking gel. The 10% gels were formed in the presence of 0.5 mg/ml recombinant MAPK-activated protein kinase 2 (MAPKAPK2) catalytic domain (residues 46–400) for assay of p38-MAPKs or 0.5 mg/ml recombinant c-Jun transactivation domain (residues 1–135) for assay of SAPKs/JNKs. In gel assays were performed as previously described [6,11] except that, during the phosphorylation, gels were incubated for 1 h with 25 μCi/gel [γ-^{32}P]ATP (NEN) in 10 ml 40 mM HEPES pH 8.0, 0.5 mM EGTA, 10 mM $MgCl_2$, 15 mM ATP, 0.1 mM cyclic AMP-dependent protein kinase inhibitory peptide. Gels were dried onto 3MM Whatman paper and autoradiographed, with quantification by laser scanning densitometry.

Western blots for phospho-p38-MAPK and phospho-ERK1/2 were performed with antibodies (New England Biolabs) against the dually phosphorylated (activated) species according to the manufacturer's instructions. The bands were detected using the ECL method (Amersham) with quantification by laser scanning densitometry.

MAPKAPK2 was partially purified by Mono S fast protein liquid chromatography (FPLC) and assayed as previously described [9].

3. Results

Ado (10 mM) stimulated the phosphorylation of p38-MAPK as assessed by immunoblotting (Fig. 1A). This was maximal at 5 min and declined thereafter, although it remained elevated for at least 30 min. The degree of phosphorylation was comparable to that induced by 20 min ischaemia/10 min reperfusion. Maximal activation (10-fold relative to controls, as assessed by in gel assays) of p38-MAPKs was observed at 5 min, and activity remained elevated for at least 30 min (Fig. 1B). This activation was comparable to that induced by ischaemia/reperfusion. We also examined activation of MAPKAPK2, which is phosphorylated and activated by p38-MAPK [12]. Ado (10 mM) stimulated a single peak of activity which eluted at approximately 0.18 M NaCl on Mono S FPLC (results not shown). MAPKAPK2 was activated approximately 4.5-fold relative to controls at 5 min and re-

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Abbreviations: Ado, adenosine; ERK, extracellularly responsive kinase; FPLC, fast protein liquid chromatography; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK2, MAPK-activated protein kinase 2; PMA, phorbol 12-myristate 13-acetate; SAPK, stress-activated protein kinase

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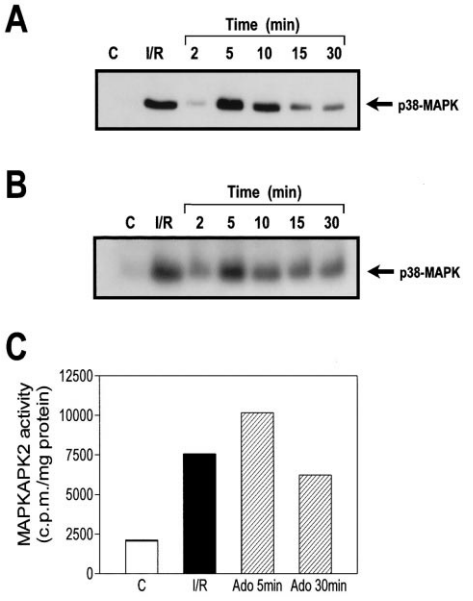

Fig. 1. Activation of p38-MAPK and MAPKAPK2 in hearts perfused with Ado. Hearts were perfused with 10 mM Ado for the times indicated, perfused under control conditions (C), or rendered ischaemic (20 min) and then reperfused (20 min) (I/R). A: p38-MAPK phosphorylation in extracts was determined using an antibody against phospho-p38-MAPK. B: The time course of p38-MAPK activation in the extracts was examined by in gel assays with recombinant MAPKAPK2(46–400) as substrate. C: MAPKAPK2 activity in hearts perfused with 10 mM Ado, as determined by Mono S FPLC. The experiment was repeated with similar results.

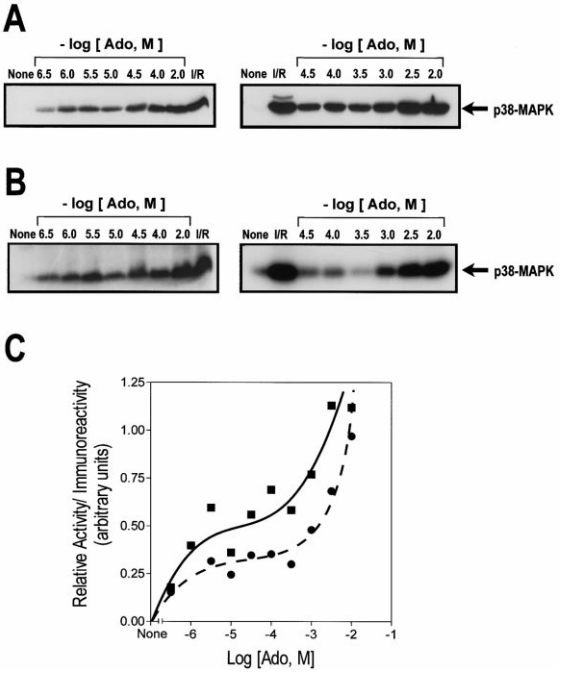

Fig. 2. Concentration-dependent phosphorylation and activation of p38-MAPK in hearts perfused with Ado. Hearts were perfused for 5 min with Ado. To assist with comparison, the separate gels contained samples at overlapping Ado concentrations. I/R, hearts rendered ischaemic for 20 min and reperfused for 10 min. A: Concentration-dependent phosphorylation of p38-MAPK by Ado as determined by immunoblotting. B: Concentration-dependent activation of p38-MAPK by Ado as determined by in gel assays. C: Quantification of p38-MAPK phosphorylation (■, solid line) and activation (●, dashed line). Results were expressed relative to the signal in ischaemic/reperfused hearts. The experiment was repeated with similar results.

mained elevated at 30 min (approximately 3-fold) (Fig. 1C). As with p38-MAPK, MAPKAPK2 activation by Ado was comparable to that in hearts subjected to ischaemia/reperfusion. The concentration-dependence of p38-MAPK phosphorylation and activation was biphasic (Fig. 2A–C). Phosphorylation (Fig. 2A) and activation (Fig. 2B) was detectable at 0.3 μM and increased over a narrow concentration range (0.3–3.0 μM, Fig. 2C). Following a plateau (10 μM–0.3 mM), there was a second marked increase that had not saturated at 10 mM Ado.

Ado (10 mM) also rapidly activated both p46 and p54 SAPKs/JNKs (maximal 6-fold activation relative to controls at 5 min) with activities remaining elevated for 30 min (Fig. 3A). As with p38-MAPK, activation of the SAPKs/JNKs by Ado was comparable to that by ischaemia/reperfusion. Unlike p38-MAPK, the SAPKs/JNKs were only strongly activated by high concentrations (10 mM) of Ado (Fig. 3B).

Phosphorylation of ERK1 (44 kDa) and ERK2 (42 kDa) induced by 10 mM Ado was maximal at 5 min and remained elevated for 30 min (Fig. 4A). PMA is the most powerful activator of cardiac ERKs so far identified [13], and the response of ERKs to 10 mM Ado was about 40% of that seen in hearts perfused with 1 μM PMA in this example (Fig. 4A). The concentration dependence of ERK phosphorylation was also examined (Fig. 4B) and displayed a biphasic pattern (Fig. 4C) similar to that seen for p38-MAPK (Fig. 2C).

4. Discussion

We and others have shown that p38-MAPK and its substrate MAPKAPK2 are strongly activated by myocardial ischaemia and their activation is maintained on reperfusion [6,7]. SAPKs/JNKs are strongly activated during the reperfusion phase [5–7]. The activation of these MAPKs may be partly mediated by the release of reactive oxygen species. Exogenous H_2O_2 activates MAPKs in perfused heart [9], and the effects of ischaemia/reperfusion on MAPKs can be reduced by antioxidants [9]. Analogous effects have been ob-

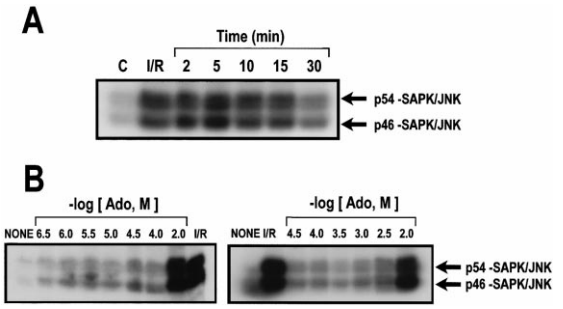

Fig. 3. Activation of SAPKs/JNKs in hearts perfused with Ado. A: Hearts were perfused with 10 mM Ado for the times indicated and SAPK/JNK activity was determined by in gel kinase assays with c-Jun(1–135) as substrate. B: Concentration-dependent activation of SAPKs/JNKs by Ado as determined by in gel assays. Hearts were perfused for 5 min with Ado. To assist with comparison, the separate gels contained samples at overlapping Ado concentrations. I/R, hearts rendered ischaemic for 20 min and reperfused for 10 min. The experiment was repeated with similar results.

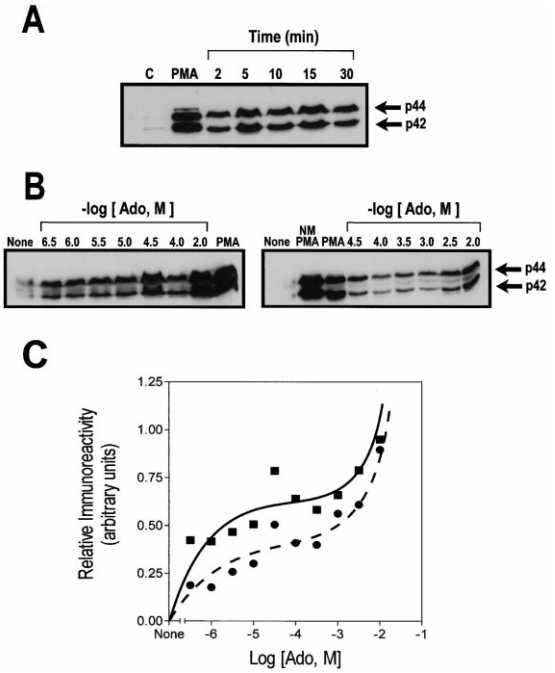

Fig. 4. Phosphorylation of ERKs in hearts perfused with Ado. A: Hearts were perfused with 10 mM Ado for the times indicated and ERK phosphorylation (p44: ERK1; p42: ERK2) was determined by immunoblotting. B: Concentration-dependent phosphorylation of ERKs (p44: ERK1; p42: ERK2) by Ado as determined by immunoblotting. Hearts were perfused for 5 min with Ado. To assist with comparison, the separate gels contained samples at overlapping Ado concentrations. Samples from hearts perfused with PMA (1 μM, 5 min) were included for comparative purposes. The right hand panel of B also shows a marker sample prepared from neonatal rat cardiac myocytes in primary culture exposed to 1 μM PMA for 5 min. C: Quantification of ERK1 (■, solid line) and ERK2 (●, dashed line) phosphorylation. Results were expressed relative to the signal in PMA-perfused hearts. The experiment was repeated with similar results.

served in cardiac myocytes [14–17]. Since exogenous Ado is a powerful preconditioning agent [1], we tested its effects on the activation of three MAPK subfamilies.

MAPKs are activated by dual phosphorylation of a Thr-Xaa-Tyr motif by dual-specificity MAPK kinases. Here, we have examined phosphorylation (p38-MAPK and ERKs) or activation (p38-MAPK and SAPKs/JNKs), the two processes being equivalent. For all MAPKs, Ado (10 mM) induced rapid phosphorylation and/or activation that was maximal within 5 min (Fig. 1AFig. 3AFig. 4A). For p38-MAPK and SAPKs/JNKs, the activation was comparable to that induced by ischaemia/reperfusion. For ERKs, the response was approximately 40% of that induced by PMA. These treatments powerfully activate the respective MAPKs [6,13]. MAPKAPK2 was also powerfully activated by 10 mM Ado (Fig. 1C). p38-MAPKs (Fig. 2) and ERKs (Fig. 4B,C) both showed a biphasic dependence of activation on Ado concentration with one phase saturating at around 10 μM and a second phase that was activated by higher concentrations. This suggests the existence of two processes. The high affinity process might be Ado receptor-mediated, whereas the low affinity process might result from deamination of Ado to hypoxanthine and oxidation of hypoxanthine by xanthine oxidase to produce reactive oxygen species, which are known activators of cardiac MAPKs [9,14,16,17].

Our results may have relevance to cardiac ischaemic preconditioning [2] for which two phases have been characterised: an early phase that lasts only 1–3 h before the heart is again fully susceptible to ischaemic damage, and a late phase that appears after about 24 h and is thought be caused by changes in gene and protein expression [3,4]. In addition to ischaemia, preconditioning can be induced in hearts or cardiac myocytes by exogenous Ado or with other Ado receptor agonists [1]. Ado may be an endogenous mediator of preconditioning in a number of species [1,3,4] although whether this is true for rat is controversial [18,19]. Preconditioning can also be induced by protein kinase C activation [20–23], reactive oxygen species [24,25], α_1-adrenergic agonists [26], endothelin-1 [27] and bradykinin [28]. All of these activate MAPKs in the heart, frequently through protein kinase C-dependent mechanisms [9,13,14,16,17,29–32]. The current thinking is that activation of protein kinase C and/or opening of ATP-sensitive K^+ channels may be involved [3]. However, our data suggest that the involvement of MAPKs in preconditioning is worthy of further investigation.

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